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The role of clock genes and rhythmicity in the liver

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The liver is the important organ to maintain energy homeostasis of an organism. To achieve this, many biochemical reactions run in this organ in a rhythmic fashion. An elegant way to coordinate the temporal expression of genes for metabolic enzymes relies in the link to the circadian timing system. In this fashion not only a maximum of synchronization is achieved, but also anticipation of daily recurring events is possible. Here we will focus on the input and output pathways of the hepatic circadian oscillator and discuss the recently found flexibility of its circadian transcriptional networks.

1. Introduction

The liver maintains energy homeostasis in mammals (Voet et al., 2008). This organ is divided into multiple lobes and is composed to the main part (70–80%) of a singular cell type, the hepatocyte. Situated nearly in the center of the body, the liver is well connected to the vascular system enabling an easy flux of compounds through this organ. Probably the major biochemical reaction in the liver is the breakdown of glucose to smaller carbohydrates and energy via the Krebs cycle. Gluconeogenesis reverses this reaction if there is sufficient energy available, and the excess of glucose is stored as glycogen deposits in the liver. Due to the rhythmic storage and degradation of glycogen, the content of liver glycogen fluctuates daily between 1% and 10% of the total liver mass (Schmutz et al., 2010).

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The glucose metabolism is linked to the fatty acid metabolism via acetyl-coenzyme A (Voet et al., 2008). Fatty acids are stored as triacylglycerols and as such represent another way to store metabolic compounds and energy. Amino acids are taken up from the blood and are used to synthesize proteins, or they are broken down to pyruvate and urea. This pyruvate is then a substrate for gluconeogenesis. The metabolic performance of the liver, however, should be related to the overall physiology of the entire organism (Fig. 1). The brain is the major consumer of glucose and ketone bodies secreted by the liver, but other tissues such as the muscles rely on a steady supply of these compounds as well. The muscles also store energy in the form of glycogen, but in contrast to the liver the glucose stays in the muscle cells. The huge need for energy of the muscles is satisfied by an additional supply of fatty acids. Finally, in the need of more energy, muscle cells start to degrade proteins and the liver takes up the left over amino acids in the form of alanine and glutamine and recycles them. Fatty acids and triglycerides are transported from the liver to the more specialized adipose tissue for long-term storage. White adipose tissue is storing fat,

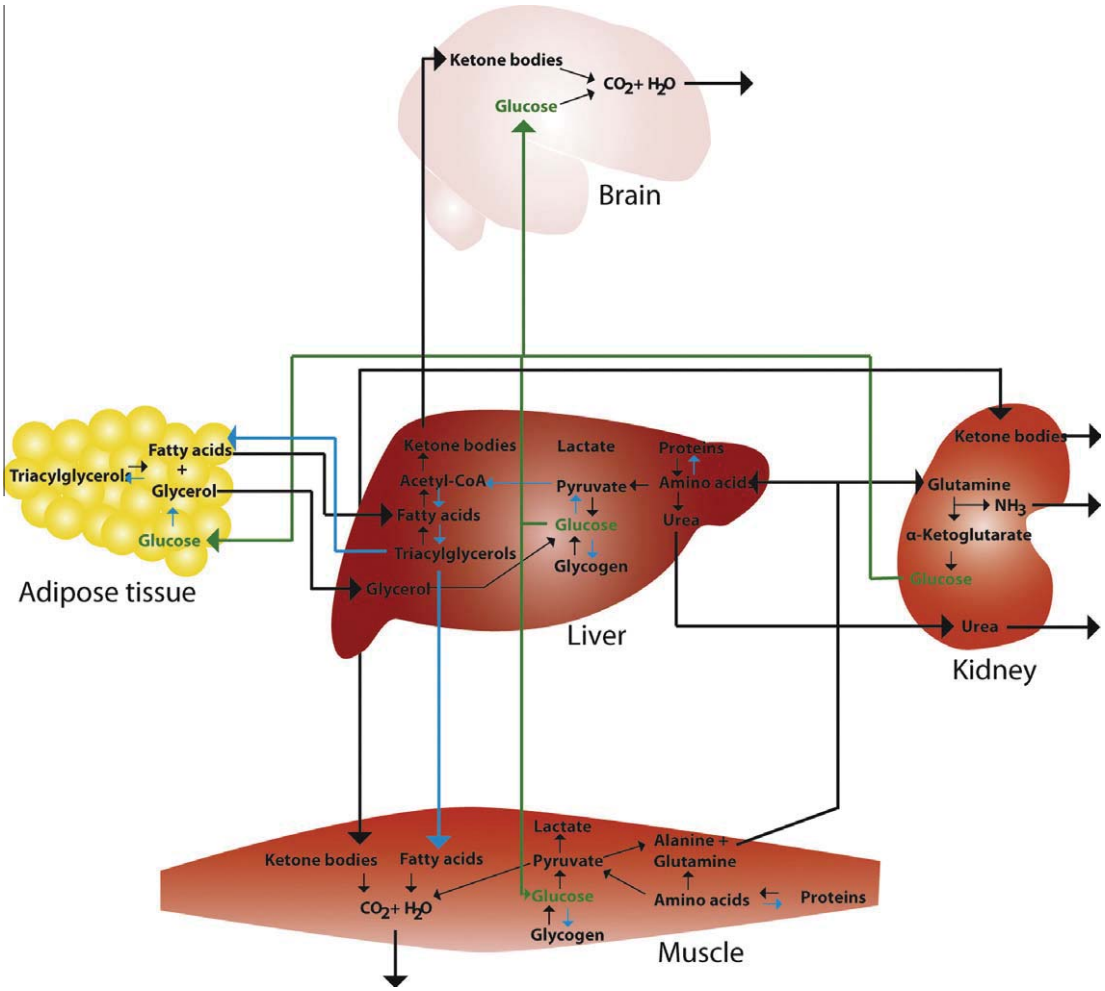

Fig. 1. Metabolic fluxes between the organs maintain homeostasis. The liver and the kidney secrete glucose, which is taken up by other organs such as brain, adipose tissue, and muscles. However, only the liver has the potential to store and secrete glucose to maintain constant blood glucose concentration. Other pathways in the liver regulate the lipid metabolism and the production of ketone bodies. Fatty acids are either transported to and stored in the adipose tissue as triacylglycerols, or used by the muscles. The ketone bodies provide extra energy to the kidneys, brain and muscles. All compounds that are in excess (e.g. urea) are excreted via the kidneys. Green arrows: flux of glucose; blue arrows: pathways that predominate in the well-fed state; black arrows: flux of other compounds. Adapted from Voet et al. (2008).

whereas brown adipose tissue generates heat to regulate body temperature. Finally, everything that is in excess or no longer useful for the liver (e.g. urea) is transported to the kidneys to become excreted.

In the view of these complicated metabolic networks, it is not surprising that there must be some kind of coordination of these processes. Many coupled biochemical reactions just run in the favorable direction according to the mass action law. However, it would be advisable to separate anabolic and catabolic reactions such as the Krebs cycle and gluconeogenesis to avoid unnecessary interference between the two pathways. This will lead to higher efficiencies of both pathways. Easy ways to uncouple such reactions are to separate them either locally, i.e. in different compartments of the cell, or temporally. The latter phenomenon can be achieved by the rhythmic expression of enzymes involved in the anabolic or catabolic reactions. A recent study revealed that up to 15% of the liver transcriptome is expressed in a rhythmic fashion (Vollmers et al., 2009) and food availability directly drives the expression of many of these genes. However, a relatively small subset of these transcripts remained rhythmic even under fasting conditions, i.e. they are driven by the endogenous, circadian clock of the liver. What is the advantage of driving one part of the hepatic transcriptome by food availability, and the other part by the circadian clock? Coupling of the expression to food availability allows fine-tuning of the expression according to the immediate needs. Coupling of the expression to the circadian clock allows anticipation and preparation for daily recurring events (i.e. food uptake). This may ultimately contribute to the fitness of an organism. Interestingly, some factors involved in detoxification share both kinds of regulation. The xenobiotic compound sensor constitutive androstane receptor (CAR; NR1I3) is expressed in a circadian fashion in the liver (Gachon et al., 2006). However, in the presence of xenobiotic ligands, its expression is super-induced. Due to the important function of the circadian clock on liver metabolism, we will discuss here recent advances on the synchronization of circadian rhythms in the liver and on the hepatic output.

2. Synchronization of the liver oscillator by systemic timing cues

To ascertain coordinated systemic rhythms for the entire organism, all tissue clocks need to be synchronized with the environment and between each other. As 'master' pacemaker, the suprachiasmatic nucleus (SCN) is important for synchronization and the establishment of stable phase relationships between the clocks in different organs. This is underlined by the study of Yoo et al. (2004) reporting that even though *Per2-luciferase* expression

in peripheral tissues of SCN-lesioned mice is rhythmic and persists for more than 20 cycles, these oscillations are significantly phase-desynchronized in individual mice and from animal to animal (Yoo et al., 2004). Hence, the main function of the SCN is to provide stable phase relationships of the peripheral oscillators.

The liver, with its importance for metabolic processes and various physiological functions, must be able to adapt quickly to changes in the environmental cycle. Input to the liver oscillator involves systemic signals that are mostly controlled by the SCN, but also timing cues (such as feeding time) that are transmitted to the liver via other (maybe more direct) pathways. Light is the most potent timing cue or Zeitgeber of the circadian clock. However, whereas light directly resets the circadian clock in the SCN, changes in the lighting schedule are communicated to peripheral tissues via indirect output pathways emanating from the SCN (Yamazaki et al., 2000). This may explain why in comparison to the SCN oscillator peripheral clocks respond with a certain delay to changes in the lighting schedule.

Systemic entrainment of peripheral tissues, notably of the liver, by the SCN employs several ways that may function in parallel. Direct routes from the SCN involve neuronal and humoral signals. A recent study by Cailotto et al. (2009) shows by selectively removing the autonomic innervation to the liver that light input information affects gene expression in the liver via the autonomic nervous system (Cailotto et al., 2009). Comparable experiments using stimulation of the autonomous nervous system have reached a similar conclusion (Terazono et al., 2003). Regarding entrainment mechanisms by hormones, glucocorticoids play an important role. Plasma glucocorticoid concentration is regulated in a rhythmic fashion via the hypothalamus/pituitary/adrenal axis with contribution of the SCN (reviewed in Buijs et al., 2003). The glucocorticoid hormone analog dexamethasone synchronizes the oscillators of cultured cells (Balsalobre et al., 2000). Moreover, injection of dexamethasone alters the phase of clock gene expression in the liver and other peripheral tissues but not in the SCN. It appears that the differential action of glucocorticoids on peripheral tissues and on the SCN is due to the differing expression of the glucocorticoid receptor in these tissues. Glucocorticoid receptors (GRs) may directly act on the oscillator and its components as glucocorticoid response elements (GRE) were identified in several clock genes and GR occupies a GRE within the clock gene *Per2* (So et al., 2009). In addition, glucocorticoid signaling can affect up to 60% of the rhythmic liver transcriptome (Reddy et al., 2007) suggesting that glucocorticoids transmit information about the nutrient state to gene expression. Glucocorticoids appear not to be the only nuclear receptor ligands secreted in a circadian manner. Several genes involved in the steroid biosynthesis pathway in the adrenal gland are rhythmically expressed (Oster et al., 2006) and aldosterone secretion (Williams et al., 1972) as well as thyroid hormone secretion (Campos-Barros et al., 1997) have been shown to display daily rhythms. Several nuclear hormone receptors show rhythmic variations on the mRNA level in various tissues (Yang et al., 2006) and some of them bind in a rhythmic fashion to the regulatory regions of clock and metabolic genes (Schmutz et al., 2010). It is conceivable that entrainment makes use of changes in rhythmic hormone secretion that is transmitted to peripheral oscillators via altered transcriptional regulation by nuclear receptors (Yang et al., 2006).

Another direct output of the SCN is the rest/activity cycle. By controlling rest and activity, the SCN further influence body temperature. Body temperature is subject to circadian variation, and this fluctuation depends on a functional SCN (Wachulec et al., 1997). In mammals, temperature cycles contribute to the entrainment mechanisms of peripheral clocks. Schibler and colleagues demonstrated a few years ago that rhythmic temperature cycles maintain circadian cycles in fibroblasts (Brown et al., 2002). Interestingly, circadian changes in environmental temperatures were able to phase shift gene expression in the liver but not in the SCN. In addition, astrocytes have been shown to be entrainable by temperature cycles of 1.5 °C (Prolo et al., 2005). The resistance of the SCN but not peripheral clocks to temperature changes may originate from network interactions inside the SCN. In a recent study, Buhr and coworkers demonstrated by assessing *Per2-luciferase* rhythms in diverse tissues *ex vivo* that peripheral tissues are highly responsive to temperature pulses (Buhr et al., 2010). They respond with type 0 phase resetting, i.e. a kind of restart of the circadian clock from a defined point, indicating that peripheral tissues are susceptible to entraining signals. By contrast, the phase of the SCN is resistant to temperature changes, a property due to the intercellular communication between SCN neurons involving L-type calcium channels.

Temperature timing cues may be transmitted to tissue clocks via the heat-shock response pathway. A very elegant study by Kornmann and colleagues showed that rhythmic transcription of most genes in the liver depends on functional hepatocyte clocks (Kornmann et al., 2007). However, the transcription of a subset of genes oscillated in a robust fashion independent of a functional liver clock, and this rhythmic transcription appears to have been driven by systemic cues. Interestingly, several heat-shock protein (HSP) genes are among these rhythmic genes. Furthermore, the transcription factor heat shock factor 1 (HSF1) has been shown to bind in a rhythmic fashion to DNA and to determine the phase of rhythmic HSP gene expression (Reinke et al., 2008). Finally, pharmacological inhibition of HSF1 blocks temperature resetting in peripheral tissues (Buhr et al., 2010).

3. Input to the peripheral clocks transmitted by food and metabolites

Feeding time appears to play an outstanding role in adaptation mechanisms of peripheral clocks to changes in the daily environmental cycle. Restricted feeding can induce changes in the phase of circadian gene expression of the liver and other peripheral organs without affecting the phase of the rhythms in the SCN, hence uncoupling peripheral clocks from the central clock (Damiola et al., 2000; Stokkan et al., 2001). Interestingly, the liver responds more rapidly to changes in the feeding pattern than the kidney, the pancreas or the heart (Damiola et al., 2000). Regarding the importance of the liver for metabolism and food processing, this rapid adaptation of the liver oscillator to the feeding pattern and by consequence phase adjustment of various processes to the availability of food may be crucial for the well being of an organism.

Mechanisms that mediate resetting of peripheral clocks by food may involve temperature-resetting cues. Feeding that is restricted to the rest phase of an animal was shown to be associated with large temperature depressions during the active phase (Damiola et al., 2000). Furthermore, glucocorticoids may act as negative players in food resetting mechanisms (Le Minh et al., 2001). Several lines of evidence indicate that food intake itself can generate entraining signals for peripheral tissues. These signals may involve food metabolites, hormones that are secreted upon feeding and fasting, and/or the intracellular redox state ($NADH/NAD^+$ ratio) that is a direct readout of the nutritional state of a cell. Interestingly, the nicotinamide adenine dinucleotide (NAD^+)-dependent protein deacetylase SIRT1 was shown to be a regulator in the circadian clock mechanism (Fig. 2; Asher et al., 2008; Nakahata et al., 2008). Furthermore, cellular NAD^+ levels cycle with a period of 24 h (Nakahata et al., 2009; Ramsey et al., 2009). This circadian variation of NAD^+ may be regulated via the rate-limiting enzyme of mammalian NAD^+ biosynthesis, nicotinamide phosphoribosyltransferase (NAMPT) that is under circadian control. These findings point towards a tight coupling of the circadian clock and cellular

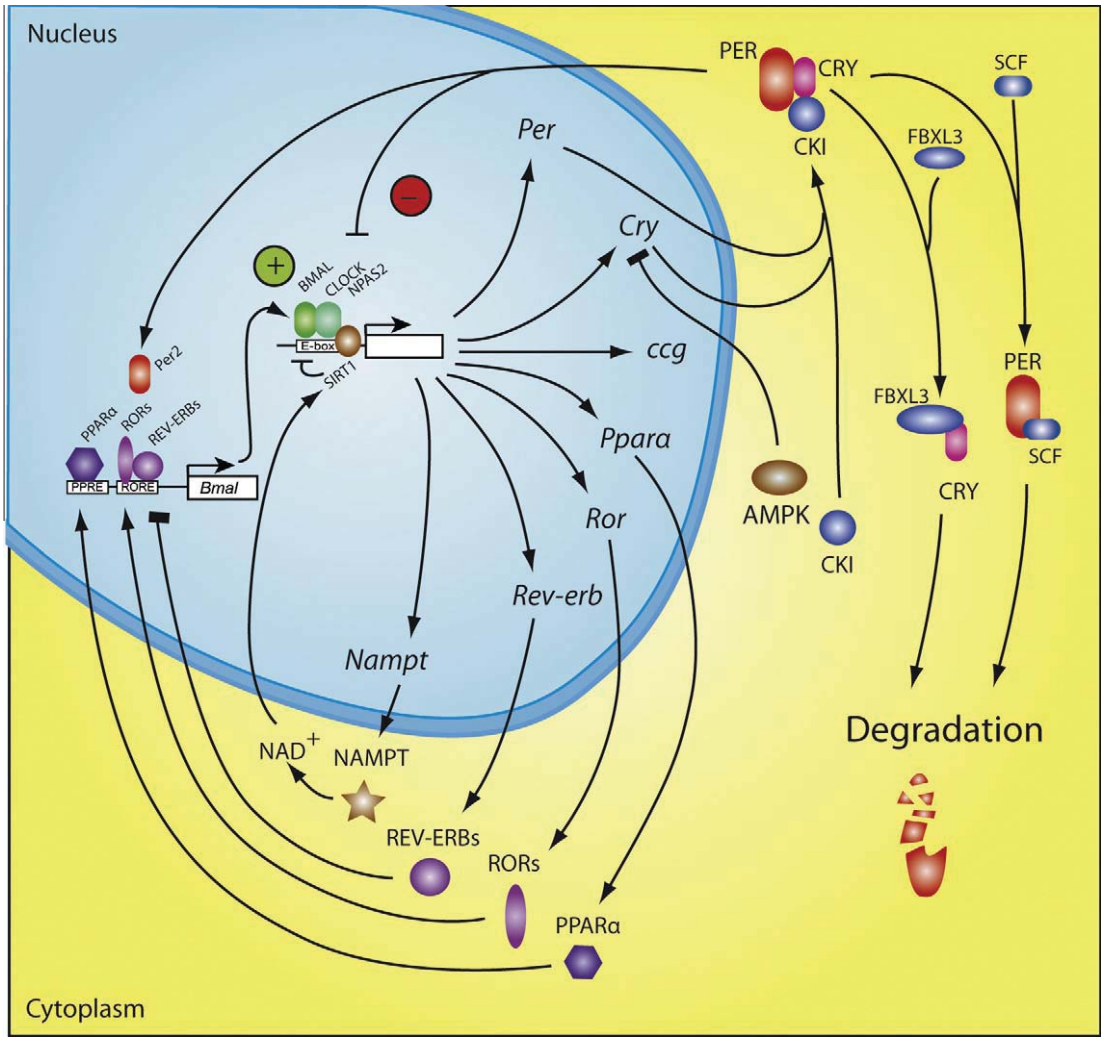

Fig. 2. The mammalian hepatic circadian oscillator. A core loop based on a feedback loop of transcriptional activation of the *Per* and *Cry* genes by BMAL1 and CLOCK (green) and subsequent repression by the accumulation of PER (red) (and CRY) proteins establishes cycles with period lengths of about a day. This loop also drives circadian expression of nuclear receptors (purple), some of which fine-tune the expression of the *Bmal1* gene in concert with the PER2 protein, and of further clock-controlled genes (ccg). The NAD^+ cycle and the NADH/NAD^+ ratio via SIRT1, and the AMP to ATP ratio via AMPK, link the circadian oscillator with the nutrition state of the cell (brown). Enzymes regulating the stability or half-lives of the repressor components (blue) are important post-translational regulators. Adapted from Maury et al. (2010).

metabolic processes in peripheral tissues. Their interplay may ascertain rapid adaptation to changes in the cellular energy state.

Recently, additional factors transmitting information about metabolic activity to the clock were identified. Asher and coworkers showed that the activity of poly(ADP–ribose) polymerase 1 (PARP-1) is rhythmic in the liver and that it is regulated by feeding (Asher et al., 2010). Interestingly, *Parp-1* knockout mice exhibit impaired food entrainment of peripheral clocks, suggesting PARP-1 as a mediator of food entrainment. Moreover, the adenosine monophosphate (AMP)-activated protein kinase (AMPK) that is a central mediator of metabolic signals was shown to be involved in the clock mechanism (Lamia et al., 2009). AMPK phosphorylates and destabilizes the core clock component CRY1 and may hence act on the oscillator mechanism (Fig. 2). Strikingly, phosphorylation of AMPK and consequently its activity appears to be regulated by nutrients and consequently by the cellular energy state via the ratio of AMP to ATP (Davies et al., 1992). Hence, AMPK may transmit nutrient signals directly to the clock by the intermediate player CRY1.

4. Transcriptional networks of the hepatic circadian oscillator

The hepatic oscillator is centered on a pair of transcriptional activators, BMAL1 (also named Mop3) (Bunger et al., 2000; Hogenesch et al., 1998) and CLOCK (Gekakis et al., 1998; King et al., 1997) and two classes of repressors of the *Period* (*Per*) (Albrecht et al., 1997; Shearman et al., 1997; Sun et al., 1997; Tei et al., 1997) and *Cryptochrome* (*Cry*) gene families (Griffin et al., 1999; Kume et al., 1999; van der Horst et al., 1999; Vitaterna et al., 1999) (Fig. 2). Briefly, BMAL1 and CLOCK activate transcription of the *Per* and *Cry* genes, whose gene products accumulate in the cytoplasm (Dibner et al., 2010; Ripperger and Brown, 2010). Upon reaching a certain threshold concentration, these repressors re-enter into the nucleus together with the CRY proteins to interfere with the activity of the transcriptional activators. Finally, when the concentration of the repressors ceases, a new transcriptional cycle of about 24 h can occur. Based on this simple model of feedback regulation, whole networks of circadian gene regulation have been constructed *in silico* (Bozek et al., 2009; Yan et al., 2008). These models suggest a very structured organization of the circadian oscillator. The core oscillator drives circadian expression of itself and a selection of direct target genes. Amongst this selection are transcriptional regulators that can amplify the action of the circadian oscillator and may provide tissue specificity. On the other hand, the rhythmic transcriptional regulators regulate target genes in a rhythmic fashion including other sets of transcription factors. In this way, a coordinated network of subsequent gene regulation is established. Due to the tight

coupling to the circadian oscillator, which generates self-sustaining, robust rhythms, these transcriptional networks persist even under constant conditions.

Very recently, a part of these models was verified by experimental data. Using the powerful method of chromatin immunoprecipitation combined with ultra-deep sequencing, Rey and coworkers have identified rhythmic binding sites for BMAL1 all over the genome of the liver (Rey et al., 2011). Not surprisingly, many known genes expressed during the late afternoon contain binding sites for BMAL1. Among the direct target genes are some enzymes involved in the glucose and triglyceride metabolism. According to the computational model, the identified targets contain a relatively high proportion of transcriptional regulators, i.e. from the nuclear receptor, bHLH, basic leucine zipper, and zinc finger families. Hence, the experimental data support a model of coordinated transcription linked to the circadian oscillator.

However, these transcriptional networks represent only a snapshot of the actual situation found in the liver. A recent paper suggests that the transcriptional networks in the liver are surprisingly flexible (Stratmann et al., 2010). The circadian clock of an organism adapts to the photoperiod. Hence, although not directly light sensitive, the circadian oscillator of the liver has to adjust to the changed environmental conditions, i.e. the overall shape of the oscillator becomes deformed to fit into the new light or dark phase. Using the circadian transcription factor *albumin D-site binding protein* (*Dbp*) gene as a model system, Stratmann et al. (2010) uncovered a mechanism that partially uncouples the expression of this gene from the circadian oscillator. The *Dbp* gene has been identified as a direct target for rhythmic BMAL1 and CLOCK binding (Kiyohara et al., 2008; Ripperger and Schibler, 2006; Ripperger et al., 2000; Yamaguchi et al., 2000). In their study, Stratmann et al. (2010) have identified a special function of a BMAL1 binding site in the promoter region of the *Dbp* gene to adjust the phase of its expression according to the photoperiod. Since DBP and the related factors TEF and HLF regulate a variety of metabolic and detoxification enzymes prior to the food uptake of the animals (Gachon et al., 2006), this may be the rationale for this sophisticated regulation. The uncoupling mechanism provides flexibility to the phase of *Dbp* expression to ascertain that the DBP protein can activate the transcription of the metabolic and detoxification enzymes always prior to the food uptake of the organism. Hence, the anticipation of this important time point in the life of a mouse, the transition from the light to the dark (or activity) phase, is maintained, at least regarding the activity of DBP. However, it is tempting to speculate that other, equally sophisticated mechanisms exist for other transcriptional regulators as well.

5. Linking the circadian clock and metabolic pathways in the liver

Recent studies suggest an important role of the hepatic circadian oscillator in the temporal organization of carbohydrate metabolism. By taking up and storing glucose at times when it is abundant and production of glucose at times when the organism needs it, the liver is the important organ for energy and glucose homeostasis. Strikingly, hepatic processes regulating the availability of glucose, i.e. glycogen production and breakdown, as well as gluconeogenesis, show daily rhythms (Kida et al., 1980; Roesler et al., 1985; Roesler and Khandelwal, 1985). The circadian clock plays an important role in regulating these processes in a temporal manner. The two important hormones for glucose homeostasis, insulin and glucagon, display circadian rhythms and affect liver metabolism (Ruiter et al., 2003; Yamamoto et al., 1987). While glucagon increases the glucose concentration by stimulating glycogenolysis and to some extent gluconeogenesis in the liver, insulin has the opposite effect (Voet et al., 2008).

Several genes coding for key enzymes of glucose metabolism show circadian variation in the liver (Lamia et al., 2008; Panda et al., 2002; Storch et al., 2002). Importantly, the study by Lamia et al. (2008) demonstrates that mice with a liver specific deletion of *Bmal1* exhibit abnormal glucose homeostasis. Several genes important for hepatic glucose metabolism lose circadian regulation when *Bmal1* function in the liver is impaired. While some of them may be regulated via complicated circadian networks, the gene coding for glucose transporter 2 (*Glut2*) may be controlled directly by the clock component BMAL1. Maximal expression of *Glut2* is found during the fasting phase of the animal. This timed expression facilitates glucose export during periods of minimal glucose ingestion (i.e. the fasting period) and limits glucose export from the liver during the feeding phase, when glucose is abundant.

Temporal orchestration of hepatic glucose metabolism is further mediated by regulation via protein–protein interactions. The transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) is expressed in a circadian fashion and regulates the activity of nuclear receptors of the ROR family (Liu et al., 2007). This affects the expression of clock genes and metabolic target genes. The recent study by Zhang et al. (2010) suggests the clock component CRY1 as circadian regulator of hepatic gluconeogenesis (Zhang et al., 2010). By its interaction with the Gs α subunit of G proteins, CRY1 appears to modulate G protein coupled receptor signaling. This may lead to temporal regulation of glucagon signaling, which is involved in the activation of hepatic gluconeogenesis.

An elegant way of temporal coordination of hepatic metabolism was proposed by the study of Schmutz et al. (2010). They demonstrate that the clock component PER2 physically interacts with several nuclear receptors. Via these interactions, PER2 can bind to the regulatory regions of nuclear receptor target genes and function as co-regulator of nuclear receptor-mediated transcription. This type of circadian regulation may affect the expression of a variety of metabolic genes, hence strengthening and amplifying rhythmic output. Interestingly, PER2 binding is detected at the regulatory region of the *glucose-6-phosphatase* (*G6pc*3) gene, a key player in hepatic glucose metabolism. PER2 contributes to the rhythmic expression of this gene, as *G6pc3* expression was altered in $Per2^{Brdm}$ mutant mice. In summary, it appears that the liver oscillator makes use of several routes to regulate hepatic glucose metabolism and hence energy and glucose homeostasis.

6. Conclusions

The hepatic metabolism is organized in a rhythmic fashion. The circadian clock of the liver maintains one part of the rhythmicity. Since these rhythms are generated by a real clock mechanism, they can be phase-advanced in relation to their need, which allows for anticipation and preparation for daily recurring events. This may be the selective advantage to shape and to maintain these complicated clock mechanisms. Interestingly, these clock mechanisms do not remain phase-locked but are flexible to enable adjustment to the changing environment. Although mainly mediated by transcriptional and post-translational feedback loops (Fig. 2), many other ways exist to transmit oscillator information to the metabolic pathways. One recent example is the oscillator component PER2, which interacts with multiple nuclear receptors and nuclear receptor target genes. The other, major part of rhythms is driven solely by the availability of food. In this case, specific proteins have to sense the concentration of compounds and to up- regulate the corresponding metabolic or detoxification pathway. These regulatory cascades are probably similarly orga-

nized as the cascades driven by circadian clocks but they should not be self-sustaining.

Interestingly, in the mouse, food-driven rhythmic processes, e.g. the rhythmic glycogen accumulation in the liver, persist for about a day. As a result, the mouse has to replenish its energy store once a day. These pseudorhythms could form the base for a phenomenon observed even in arrhythmic mice: the food anticipatory activity (Mistlberger, 1994; Stephan, 2001). Mice and other mammals can predict the availability of food with reasonable precision, when the food is provided always at the same time of the day. The molecular mechanism of this phenomenon is currently unknown but may be related to the daily cycle of the nutrient state of the animal.

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